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Internally transposed signal sequence of carp preproinsulin retains its functions with the signal recognition particle

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It is shown that the signal sequence of carp preproinsulin is functional with the dog pancreatic signal recognition particle (SRP) both when present at its normal location at the amino-terminus of the protein or when engineered to an internal location. Inhibition of translation by SRP in the absence of microsomal membranes, reconstitution by SRP of the translocation competence of high-salt inactivated microsomes and signal peptide cleavage all occur with the signal sequence being preceded by a highly charged peptide segment of 39 amino acid residues (the distance from the amino-terminus to the cleavage site of the signal peptidase is increased to 56 residues).

Signal sequence *Signal recognition particle* *Transcription* *Translation* *Protein translocation*

1. INTRODUCTION

The SRP is known to be involved in the initiation of protein translocation across the endoplasmic reticulum membrane [1–3]. It interacts with ribosomes synthesizing proteins to be translocated and in general exerts a site-specific translational arrest, presumably when the signal sequence has emerged from the ribosome. The translational arrest is released by interaction of SRP with its receptor in the RER membrane (docking protein) [4,5]. Simultaneously, the ribosome is bound to the RER membrane and translocation commences (review [6]). SRP has therefore two functions which can be assayed separately: (i) inhibition of translation in the absence of microsomal membranes, and (ii) reconstitution of the translocation competence of K-RMs which are devoid of SRP and therefore inactive by themselves.

It appears that the hydrophobic core of a signal sequence is essential for the function of SRP [7] but further requirements are still unknown. In particular, one should like to know whether the location of a signal sequence in a polypeptide chain is of any importance. Although most signal sequences are located at the N-terminus of an exported polypeptide and cleaved off after transfer across the membrane, there are a number of cases where internal signal sequences have been implicated [8–13]. It is not yet certain that both functions of SRP are triggered by internally located signal sequences. In fact, for two membrane proteins with presumed internal signal peptides the translational arrest was weak although SRP was required for membrane incorporation [12]. The SRP-induced translational arrest is also weak for ovalbumin (P. Walter, personal communication), a secretory protein with an internally located signal sequence [13].

This paper is dedicated to Professor S.P. Datta whose outstanding contribution to science as Managing Editor of FEBS Letters will be remembered forever

Abbreviations: SRP, signal recognition particle; RER, rough endoplasmic reticulum; RM, rough microsomes; K-RM, rough microsomes washed with high salt; PMSF, phenylmethanesulfonyl fluoride

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Here we show that the signal sequence of carp preproinsulin, which is normally located at the amino-terminus, retains its functions with dog pancreatic SRP when engineered to an internal location.

2. MATERIALS AND METHODS

2.1. *Construction of plasmids*

Fig.1 shows the scheme whereby the plasmids pSW9 and pSW39 were constructed. Plasmid pKT218 [14] was used as a source of the β-lactamase promoter plus the coding region for the first 6 amino acid residues of this gene (see also table 1). The β-lactamase promoter has been shown to be suitable for in vitro transcription-translation using *E. coli* RNA polymerase and a wheat germ cell-free system [15]. To facilitate further cloning, part of the multicloning site of plasmid pEMBL9 [16] was placed after the β-lactamase region yielding plasmid pBLA22. The coding region of carp preproinsulin was obtained from plasmid pLCR665 which contains a complete cDNA copy of the mRNA [17,18]. Use was made for the cloning of the carp preproinsulin gene of a unique *Bam*HI site located at the beginning of the coding region of its signal sequence. Since it turned out that the presence of two β-lactamase promoters in the plasmid pSR9 obscured the results of in vitro transcription-translation, the fusion gene was cut out with *Hin*dIII and *Bgl*I and transferred to plasmid pBR322 from which the Amp gene had been deleted by cleavage with *Hin*dIII and *Pst*I. Plasmid pSW39 was constructed from pSW9 by insertion of a 90 bp fragment from pBR322 (position 930–1019) into the *Acc*I site of pSW9 just in front of the coding region for carp preproinsulin.

The final plasmids were checked by restriction enzyme analysis. The results show that the expected constructions were indeed obtained: pSW9 – *Pst*I (4000, 292, 132, 16), *Bam*HI (3835, 605), *Hin*dII (2944, 1336, 160); pSW39 – *Pst*I (4000, 382, 132, 16), *Bam*HI (3835, 695), *Hin*dII (2944, 1586). (In parentheses the obtained fragments in bp are given.)

The techniques and conditions used for DNA manipulations were essentially as described by Maniatis et al. [19]. Restriction enzymes and T_4 DNA ligase were either obtained from Boehringer Mannheim or from ZIMET Jena. T_4 DNA polymerase was purchased from Boehringer Mannheim.

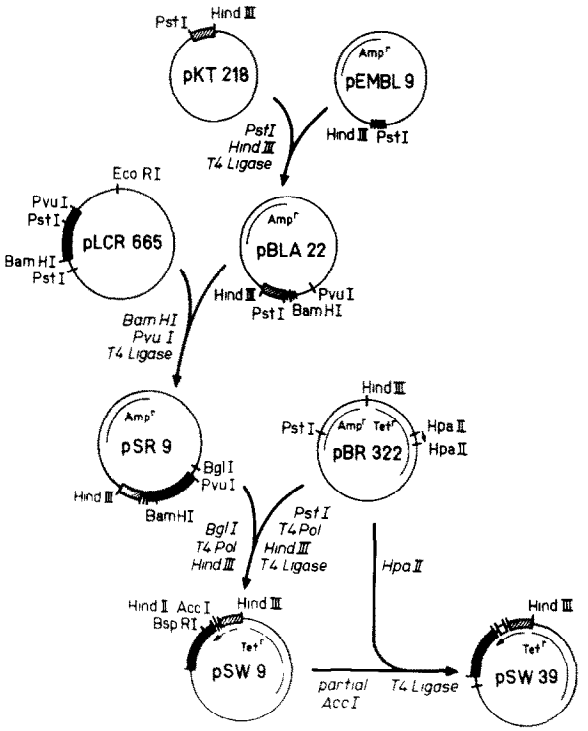


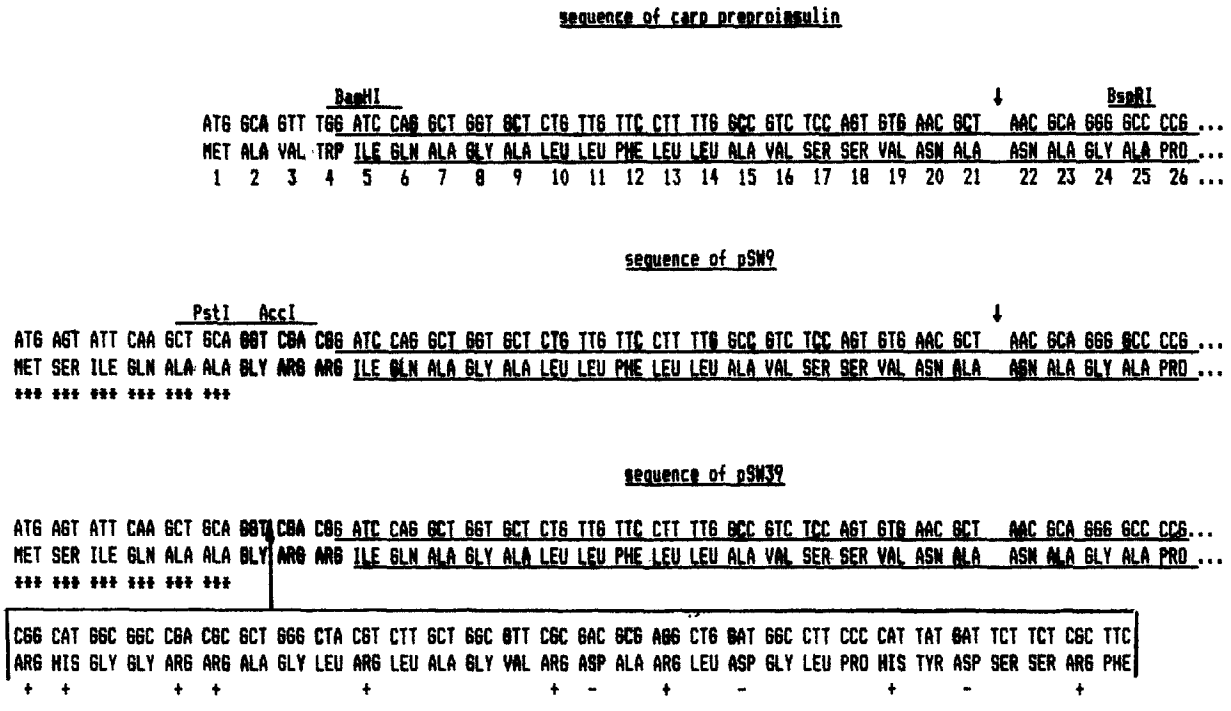
Fig.1. Scheme of construction of the plasmids pSW9 and pSW39. The *Pst*I-*Hin*dIII fragment of plasmid pKT218 [14] was isolated by electrophoresis in low melting agarose and ligated into plasmid pEMBL9 [16] which had been cleaved with *Pst*I and *Hin*dIII. The resulting plasmid pBLA22 was cleaved with *Bam*HI and *Pvu*I and a *Bam*HI-*Pvu*I fragment from plasmid pLCR665 [17,18] was inserted yielding plasmid pSR9. This plasmid was cleaved with *Bgl*I, the ends made blunt with T_4 DNA polymerase (T_4 pol) and the DNA further cleaved with *Hin*dIII. The fragment was ligated into pBR322 which had been treated in sequence with *Pst*I, T_4 DNA polymerase and *Hin*dIII. This yielded plasmid pSW9, from which plasmid pSW39 was constructed by insertion of an *Hpa*II-*Hpa*II fragment of pBR322 (positions 930–1019) into the *Acc*I site just in front of the preproinsulin gene. The hatched and black areas of the plasmids indicate the β-lactamase and carp preproinsulin regions, respectively.

2.2. *In vitro transcription-translation*

The plasmids were transcribed in vitro with *E. coli* RNA polymerase (Boehringer Mannheim) exactly as described previously, except that the capping nucleotide was omitted [15]. Translation was

Table 1

Partial sequence of carp preproinsulin and its derivatives coded by pSW9 and pSW39



The nucleotide and corresponding amino acid sequences of authentic carp preproinsulin [18] and of the constructed derivatives are shown. Underlined residues in pSW9 and pSW39 are identical with the authentic ones. The first residues in these plasmids (underlined with asterisks) correspond to those of the β-lactamase. Linker residues are indicated in bold face. The insert of plasmid pSW39 introduced into the *Acc*I site codes for a highly charged amino acid sequence. Restriction enzyme recognition sites used for cloning are indicated. The arrow shows the cleavage site of the signal peptidase [28]

carried out in a wheat germ cell-free system in the presence of [^{35}S]cysteine (New England Nuclear). SDS gel electrophoresis (12–22% linear acrylamide gels) and fluorography were performed as described [15,20]. Cell-free synthesis of authentic carp preproinsulin with poly(A)-RNA from Brockmann bodies of the carp [21] and immunoprecipitation of insulin-related polypeptides were performed as described earlier except that protein A-Sepharose was used to collect the antigen-antibody complexes [20].

Posttranslational proteolysis was carried out as follows: after translation 2 mM tetracaine, 1 mM $CaCl_2$ and 0.1 mM puromycin were added and the mixture was incubated for 8 min at 22°C. Then trypsin and chymotrypsin (0.6 mg/ml each) were added and the incubation continued at 4°C for 60 min. Detergent (0.5% Triton X-100) was added to some samples. Proteolysis was terminated by Trasylol (2000 units/ml) and PMSF (2 mM) and the products were precipitated by trichloroacetic acid before SDS gel electrophoresis.

2.3. *Test for the functions of SRP*

SRP was isolated from dog pancreas and purified by hydrophobic chromatography and sucrose gradient centrifugation [22]. K-RMs were obtained by repeated washing of RMs with 0.5 M potassium acetate. The concentrations of SRP and of K-RMs were determined by preprolactin translation and are expressed in units and equivalents (eq.), respectively, as defined in [1].

3. RESULTS AND DISCUSSION

Fig.2 shows that dog pancreatic SRP functions with authentic carp preproinsulin in the same manner as has been described before for other secretory and membrane proteins [1,4,12,23–26]. In the absence of microsomal membranes, SRP caused an inhibition of translation of preproinsulin mRNA (lanes 2–6). If K-RMs were added together with SRP, signal peptide cleavage occurred and the translational arrest exerted by SRP alone was released (lanes 8–11). K-RMs were inactive in translocation by themselves since they are devoid of SRP (lane 7). Thus, both functions of SRP, i.e. inhibition of translation and initiation of translocation, are observed with the signal sequence at its normal amino-terminal position in the polypeptide chain.

To test the influence of an internal location of the signal sequence in the polypeptide chain, two plasmids were constructed which contain the carp preproinsulin coding region under the control of the β-lactamase promoter (see fig.1 and table 1). In plasmid pSW9 the nucleotide sequence coding for the first 4 amino acid residues of the signal peptide of carp preproinsulin was replaced by that coding for the first 6 residues of the pre-β-lactamase and for 3 residues of a linker region (table 1). Plasmid pSW39 contains an additional 90 nucleotides in front of the sequence coding for carp preproinsulin so that a fusion polypeptide is expected in which the signal sequence is preceded by 39 amino acid residues (table 1). Note that the amino acid sequence in front of the signal sequence contains many charged residues.

mRNA synthesized from pSW9 or pSW39 and translated in the wheat germ cell-free system directed the synthesis of carp insulin-related polypeptides of the expected molecular masses (fig.3). It should be noted that the respective proteins were the major translation products even without immunoprecipitation (lanes 2 and 3). These data provide evidence for correct initiation of translation.

Figs 4 and 5 demonstrate that SRP exerts a translational arrest with mRNA obtained from

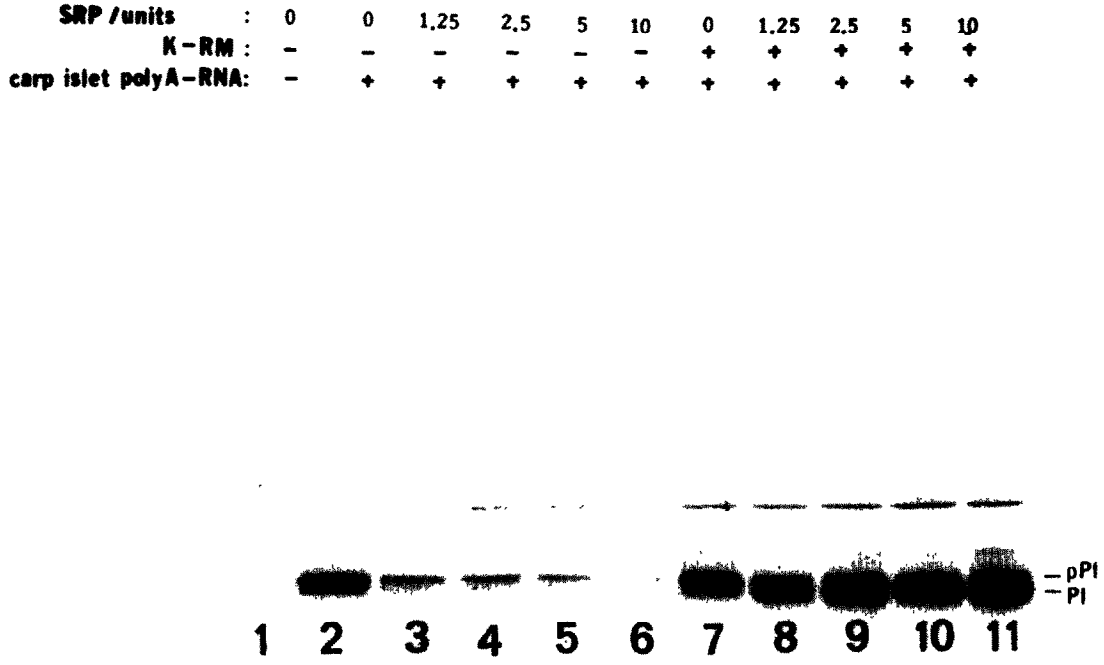


Fig.2. SRP functions with the authentic signal sequence of carp preproinsulin. Poly(A)-RNA from carp islets (about 100 ng) was translated in a wheat germ cell-free system (25 μl). Dog pancreatic SRP and/or K-RMs (1 eq.) were present during translation as indicated. The products were separated in an SDS gel and visualized by fluorography. pPI and PI denote preproinsulin and proinsulin, respectively.

Fig.3. In vitro transcription-translation of plasmids pSW9 and pSW39 yields immunoreactive products. DNA of the plasmids pSW9 (lanes 2–4) and pSW39 (lanes 5–7) was transcribed in vitro with *E. coli* RNA polymerase and the transcripts translated in a wheat germ cell-free system. The products were either applied directly to SDS gel electrophoresis (lanes 1,2,5) or first immunoprecipitated with antibodies directed against carp insulin (lanes 3,4,6,7). Controls were performed without polymerase (lane 1) and with antibodies which were presaturated with a large excess of unlabelled carp insulin (lanes 4 and 7). PPI and PI denote the positions of authentic preproinsulin and proinsulin, respectively, which served as reference.

Fig.4. SRP functions with translation products coded by plasmid pSW9. Transcripts of the plasmid pSW9 were translated in the presence of SRP and K-RMs as indicated. The products were separated on an SDS gel and visualized by fluorography. Lane 1 shows a control with no polymerase added to the transcription mixture. For the sample shown in lane 9 cycloheximide (1 mM) and methionine (1 mM) were added after 60 min of translation and the incubation was continued in the presence of SRP and K-RMs.

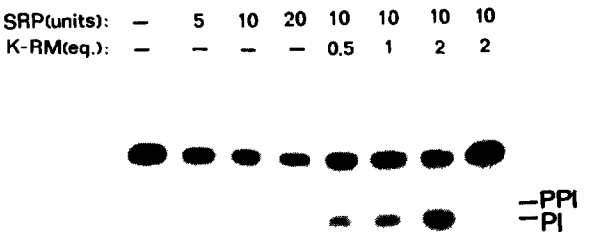


Fig.5. SRP functions with translation products coded by plasmid pSW39. Transcripts of plasmid pSW39 were translated in the presence of SRP and K-RMs as indicated. For the sample shown in the right-most lane, SRP and K-RMs were added posttranslationally (cf. lane 9 of fig.4).

both plasmids. A deletion mutant in which the signal sequence was absent (deletion of an *Acc*I-*Bsp*RI fragment, see table 1) did not show this effect (not shown). Quantitation of a number of experiments showed that the translational inhibition exerted by SRP was about equal for N-terminal and internal signal sequences. Thus, it appears that the lack of a translational arrest for some proteins [12,13] is not caused per se by an internal location of the signal sequence, in agreement with recent results obtained by mathematical modelling of the translocation process (Rapoport, T.A., Heinrich, R. and Schulmeister, T., unpublished).

If both K-RMs and SRP were present in the cell-free translation system, a partial processing was observed with the formation of a polypeptide corresponding in size to authentic carp proinsulin (figs 4 and 5). The processed material was immunoreactive with antibodies to carp insulin (not shown). It should be noted that processing of the precursor coded by plasmid pSW39 (fig.5) was reproducibly less efficient than processing of the precursor coded by pSW9 (fig.4) (see also fig.6). Since the translational arrest by SRP was approximately the same for both proteins, it is likely that the interaction of the signal sequence with SRP was unaffected by the internal location of the peptide (see above). It appears that either the docking of SRP to the membrane or, more likely, the translocation process itself, was less efficient.

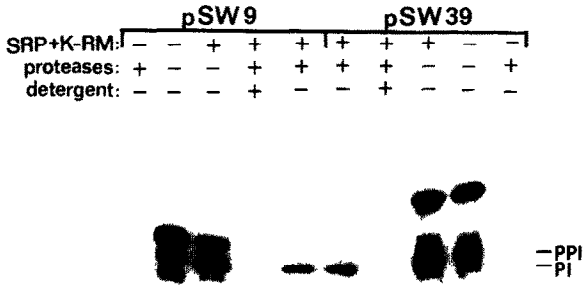


Fig.6. The processed translation products coded by pSW9 and pSW39 are translocated across the microsomal membrane. Transcripts of pSW9 and pSW39 were translated in a wheat germ system in the presence of SRP (10 units/25 μl) and K-RMs (2 eq.). Posttranslational proteolysis was carried out as described in section 2.

To prove that the processing is connected with protein translocation across the microsomal membrane, posttranslational proteolysis was carried out (fig.6). Proinsulin synthesized in the presence of SRP and K-RMs was largely resistant to proteases unless detergent was added to disrupt the membranes. On the other hand, nontranslocated precursor molecules coded by pSW9 and pSW39 were accessible to proteases even in the absence of detergent.

The data show that the signal sequence remained functional with SRP and other components of the translocation system even if placed at an internal location where it is preceded by a highly charged peptide segment. Signal peptide cleavage also occurred with the internal position of the sequence. Since processing was not observed with posttranslationally added microsomes and no processing intermediates were seen with membranes added cotranslationally (figs 4 and 5), we conclude that the signal sequence was internal during its functioning. The easiest way to envision the mechanism of translocation is a loop model [27] according to which the charged N-terminus would remain in the cytoplasm whereas the C-terminal part would be translocated across the membrane. Since the translocation process was incomplete and since the cleaved-off N-terminal part could not be detected we cannot, however, rule out that the N-terminus was also translocated.

The results are in agreement with a hypothesis previously proposed according to which the first accessible stretch (from the N-terminus) of at least 6–7 contiguous hydrophobic amino acid residues acts as a signal for translocation [7].

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REFERENCES

[1] Walter, P., Ibrahimi, I. and Blobel, G. (1981) J. Cell Biol. 91, 545–550.
[2] Walter, P. and Blobel, G. (1981) J. Cell Biol. 91, 551–556.
[3] Walter, P. and Blobel, G. (1981) J. Cell Biol. 91, 557–561.
[4] Meyer, D.I., Krause, E. and Dobberstein, B. (1982) Nature 297, 647–650.
[5] Gilmore, R., Walter, P. and Blobel, G. (1982) J. Cell Biol. 95, 470–477.
[6] Rapoport, T.A. and Wiedmann, M. (1985) Curr. Top. Membranes Transp. 24, 1–64.
[7] Finkelstein, A.V., Bendzko, P. and Rapoport, T.A. (1983) FEBS Lett. 161, 176–179.
[8] Garoff, H., Frischauf, A.M., Simons, K., Lehrach, H. and Delius, H. (1980) Nature 288, 236–241.
[9] Braell, W.H. and Lodish, H.F. (1981) J. Biol. Chem. 256, 11337–11344.
[10] Goldmann, B.M. and Blobel, G. (1981) J. Cell Biol. 90, 236–242.
[11] Holland, E.C., Leung, J.O. and Drickamer, K. (1984) Proc. Natl. Acad. Sci. USA 81, 7338–7342.
[12] Anderson, D.J., Mostov, K.E. and Blobel, G. (1983) Proc. Natl. Acad. Sci. USA 80, 7249–7253.
[13] Tabe, L., Krieg, P., Strachan, R., Jackson, D., Wallis, E. and Colman, A. (1984) J. Mol. Biol. 180, 645–666.
[14] Talmadge, K., Stahl, S. and Gilbert, W. (1980) Proc. Natl. Acad. Sci. USA 77, 3369–3373.
[15] Wiedmann, M., Huth, A. and Rapoport, T.A. (1984) Nature 309, 637–639.
[16] Dente, L., Cesareni, G. and Cortese, R. (1983) Nucleic Acids Res. 11, 1645–1656.
[17] Liebscher, D.H., Coutelle, C., Rapoport, T.A., Hahn, V., Rosenthal, S., Prehn, S. and Williamson, R. (1980) Gene 9, 233–246.
[18] Hahn, V., Winkler, J., Rapoport, T.A., Liebscher, D.H., Coutelle, C. and Rosenthal, S. (1983) Nucleic Acids Res. 11, 4541–4552.

[19] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY.
[20] Rapoport, T.A., Thiele, B., Prehn, S., Marbaix, G., Cleuter, Y., Hubert, E. and Huez, G. (1978) Eur. J. Biochem. 87, 229–233.
[21] Prehn, S., Tsamaloukas, A. and Rapoport, T.A. (1980) Eur. J. Biochem. 107, 185–195.
[22] Walter, P. and Blobel, G. (1983) Methods Enzymol. 96, 682–691.
[23] Stoffel, W., Blobel, G. and Walter, P. (1981) Eur. J. Biochem. 120, 519–522.
[24] Anderson, D.J., Walter, P. and Blobel, G. (1982) J. Cell Biol. 93, 501–506.
[25] Erickson, A.H., Walter, P. and Blobel, G. (1983) Biochem. Biophys. Res. Commun. 115, 275–280.
[26] Bassuener, R., Wobus, U. and Rapoport, T.A. (1984) FEBS Lett. 166, 314–320.
[27] Inouye, M. and Halegoua, S. (1980) Crit. Rev. Biochem. 7, 339–371.
[28] Makower, A., Dettmer, R., Rapoport, T.A., Knospe, S., Behlke, J., Prehn, S., Franke, P., Etzold, G. and Rosenthal, S. (1982) Eur. J. Biochem. 122, 339–345.